Coevolution of Host and Virus: The Pathogenesis of Virulent and Attenuated Strains of Myxoma Virus in Resistant and Susceptible European Rabbits

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Myxoma virus was introduced into the European rabbit population of Australia in 1950. Although the virus was initially highly lethal in rabbits, there was rapid selection for less virulent strains of virus and innately resistant rabbits. To investigate the basis of resistance to myxoma virus, we have compared the pathogenesis of the virulent strain of myxoma virus originally released into Australia and an attenuated, naturally derived field strain of myxoma virus. This was done in laboratory rabbits, which have not been selected for resistance, and in wild rabbits that have developed significant resistance. Wild rabbits were able to recover from infection with virus that was always lethal in laboratory rabbits. Laboratory rabbits were able to control and recover from infection with attenuated virus. This virus caused a trivial disease in wild rabbits. There was little difference between laboratory and wild rabbits in titers of either virulent or attenuated virus in the skin at the inoculation site. However, resistant wild rabbits had a 10- to 100-fold lower titer of virulent virus within the lymph node draining the inoculation site and controlled virus replication in tissues distal to the draining lymph node. Replication of virus in lymphocytes or fibroblasts cultured from wild and laboratory rabbits demonstrated that resistance was not due to altered cellular permissivity for replication. Neutralizing antibodies were present in both susceptible and resistant rabbits, suggesting that these have no significant role in resistance. We hypothesise that resistance is due to an enhanced innate immune response that allows the rabbit to mount an effective cellular immune response.

Key Words: myxoma virus; pathogenesis; rabbits; resistance; coevolution.

INTRODUCTION

Infectious diseases have the potential to drive the evolution of their hosts particularly when first introduced into a naive host species. The pathogen may also evolve to adapt to the new host. The best-documented studies of the coevolution of a pathogen with a new mammalian host species are from the release of myxoma virus into the Australian wild rabbit population. However, despite very detailed studies of the development of resistance in the rabbit and attenuation of the virus, very little is understood about the mechanisms of either resistance or attenuation (Anderson and May, 1982; Fenner, 1983; Kerr and Best, 1998). This paper details studies on the basis of resistance to myxoma virus in Australian wild rabbits.

Myxoma virus is a poxvirus (genus Leporipoxvirus) naturally found in the jungle rabbit of South America (Sylvilagus brasiliensis) or in the brush rabbit (S. bachmani) of North America. The virus is passively transmitted by mosquitoes or other biting arthropods, and in its natural hosts, it causes a cutaneous fibroma restricted to the site of inoculation. However, European rabbits (Oryctolagus cuniculus) infected with myxoma virus develop the lethal, disseminated disease myxomatosis (Fenner and Ratcliffe, 1968). The European rabbit is a major pest species in Australia and, in an attempt at biological control, the highly virulent Standard Laboratory (Moses) Strain (SLS) of myxoma virus, originally isolated in Brazil, was introduced into Australia in 1950. The results were spectacular. Following its introduction, the virus spread rapidly across the continent dramatically reducing rabbit numbers and becoming endemic in the rabbit population.

SLS initially was lethal in >99% of infected rabbits. However, there was rapid selection for less virulent strains of virus. Rabbits infected with these strains had a longer survival time although they still killed ≤90% of infected rabbits. Virus isolates from the field were graded on a virulence scale of 1–5 based on the survival rate and average survival times of small groups of infected laboratory rabbits (Fenner and Marshall, 1957). Grade 1 viruses killed essentially 100% of infected rabbits with an average survival time of <13 days, grade 3 viruses killed 70–90% of infected rabbits with an average survival time of 17–28 days, and grade 5 viruses killed <50% of infected rabbits. The strains of myxoma virus of intermediate (grade 3) virulence came to predominate in the field as these were more efficiently transmitted by mosqui-
toes because the infected rabbits survived longer with virus in the epidermis at or above the threshold titer for efficient transmission (Fenner et al., 1956).

The emergence of attenuated virus strains facilitated the natural selection of rabbits with enhanced innate resistance to myxoma virus. These resistant rabbits rapidly came to dominate rabbit populations in Australia. As an example, the survival rate of rabbits collected from a single field site and challenged with a standard grade 3 strain of myxoma virus was increased from 10 to 74% within 7 years of the first epidemic of myxomatosis (Marshall and Fenner, 1968). A similar pattern of coevolution of virus and host was observed following the release of myxoma virus in Europe (Fenner and Ross, 1994).

The pathogenesis of myxomatosis in laboratory rabbits was described by Fenner and Woodroffe (1953). Virus spreads from the initial replication site in the skin to the lymph node draining the inoculation site and then disseminates to other tissues such as spleen, lung, and testis and to secondary sites in the skin and mucocutaneous junctions. Histologically the infected tissues show a combination of cellular proliferation and cell death (Hurst, 1937). However, the histological damage in critical tissues is not sufficient to explain the lethality of the virus (Mims, 1964). Late in infection rabbits may develop acute bacterial infections of the conjunctivae and upper respiratory tract and these secondary infections have been postulated as a cause of death (Hobbs, 1928). However, in acute infections animals may die without showing signs of significant secondary infection (Fenner and Ratcliffe, 1965).

Like other poxviruses, myxoma virus encodes proteins that modulate the immune response of the host. These virulence factors include: a tumour necrosis factor binding protein (Upton et al., 1991), a gamma interferon binding protein (Upton et al., 1992), chemokine binding proteins (Graham et al., 1997; Lalani et al., 1997), and proteins such as M11L (Graham et al., 1992) and the serine protease inhibitors Serp 1, which significantly reduce the expression of MHC-1 molecules on CD4 on infected lymphocytes (Barry et al., 1992) and Serp 2, an inhibitor of intereukin-1β converting enzyme (Messud-Petit et al., 1998). At least four virulence genes encode proteins that are critical for virus replication in lymphocytes in vitro (Macen et al., 1996; Mossman et al., 1996; Barry et al., 1997). In addition, myxoma virus infection downregulates expression of MHC-1 molecules on the surface of infected cells (Boshkov et al., 1992) and CD4 on infected lymphocytes (Barry et al., 1995).

Despite the emerging molecular detail about myxoma virus, there have been no studies on the mechanism of the resistance to myxoma virus that has developed in wild rabbit populations in Australia and Europe (Kerr and Best, 1998). This is important for understanding host-virus coevolution and the use of myxoma virus as a biological control agent. As a first step toward understanding resistance, we have compared the pathogene-

s of the virulent grade I SLS in wild and laboratory rabbits with the pathogenesis of the attenuated grade 5 Uriarra strain (Ur) of myxoma virus, which was derived from SLS within 2 years of the first field release (Mykyto-wycz, 1953). Ur is usually nonlethal in laboratory rabbits but causes clinically severe myxomatosis (Fountain et al., 1997). Therefore it provides a useful model to examine recovery from myxoma virus infection. This paper describes the differences in disease caused in each group of rabbits, the replication of each virus in the skin and lymphoid tissues of these rabbits, and the antibody responses to these viruses. To determine whether resistance is a function of cell permissivity for replication, we examined the replication of myxoma virus in primary cells prepared from both wild and laboratory rabbits.

### RESULTS

**Clinical disease and autopsy findings**

All laboratory rabbits inoculated with SLS or Ur and wild rabbits inoculated with SLS developed clinical myxomatosis. This was characterized by the development of a large (6-cm diameter) red, elevated, primary skin lesion at the site of inoculation followed by thickening of the eyelids and the development of swellings (secondary lesions) on the eyelids, nose, and ears and subsequently over the entire body. Conjunctival inflammation and an initially serous and later mucopurulent discharge from the nose and eyes followed, accompanied by respiratory distress and swelling and inflammation of the anogenital region. Laboratory rabbits infected with SLS were not kept beyond 10 days after inoculation as previous experience with this virus had shown that all animals died between 10 and 14 days after infection (Robinson et al., 1999). Wild rabbits inoculated with Ur had very mild signs of myxomatosis. The time of onset (Table 1), and the subsequent severity of clinical signs varied between groups. Figure 1 shows typical animals from each group with myxomatosis.

<table>
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<tr>
<th>Clinical sign</th>
<th>Laboratory rabbits</th>
<th>Wild rabbits</th>
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<tr>
<td>Pink inoculation site</td>
<td>Day 2</td>
<td>Day 2</td>
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<td>Conjunctival inflammation</td>
<td>Days 4–6</td>
<td>Days 5–7</td>
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<td>Anogenital edema</td>
<td>Day 6</td>
<td>Day 7</td>
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<td>Secondary lesions</td>
<td>Day 6</td>
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<td>Respiratory difficulty</td>
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<td>Day 9–12</td>
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<td>Lesion regression</td>
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<table>
<thead>
<tr>
<th>Time of onset</th>
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<td>Laboratory rabbits</td>
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<td>Wild rabbits</td>
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Laboratory rabbits infected with Ur developed clinical myxomatosis that was less severe than laboratory rabbits infected with SLS (Figs. 1A and 1B). The symptoms and autopsy findings were similar to those seen in wild rabbits infected with SLS. However, disseminated secondary lesions on the body were slower to develop than in wild rabbits infected with SLS and were not generalised until Day 1,5 and a scab had not begun to form over the primary lesion until Day 17. Recovery from infection was not as advanced at Day 20 in these rabbits as it was in wild rabbits infected with SLS. The size of the primary lesion was similar to SLS infected laboratory rabbits at \( \sim 6 \) cm diameter at Day 10. In wild rabbits infected with SLS and laboratory rabbits infected with Ur, shrinkage of the testes was noted late in infection, and several wild rabbits had epididymal granulomas.

Wild rabbits infected with SLS developed similar clinical signs to laboratory rabbits infected with SLS but the severity was reduced and the onset was delayed (Fig. 1C). This was particularly the case with secondary lesions, which were generally distributed over the body of laboratory rabbits by 6 days after infection but took until 8 days to be generally distributed over the bodies of wild rabbits. Similarly, respiratory distress was noted in laboratory rabbits infected with SLS by 7 days after inoculation but was delayed until 10 days in wild rabbits. One wild rabbit inoculated with SLS died between Days 10 and 11. In this rabbit, the lungs were edematous and blood filled. This was also the wild rabbit with the most extensive primary and secondary lesions. At Day 10, all the wild rabbits were depressed and had ocularnasal discharges. The primary lesions were extremely elevated (2.5–3 cm high) and domed. Laboratory rabbits had more severe clinical signs at this time including respiratory distress. At 12 days after infection all the wild rabbits were experiencing respiratory difficulties; however, the secondary and primary lesions had begun to scab over. At 15 days after infection, the breathing had improved and the scrotal oedema and anogenital inflammation were reduced; by 20 days after infection the rabbits were virtually recovered with scabbing of the primary lesion.

**FIG. 1.** Clinical appearance of rabbits with myxomatosis. Laboratory rabbits at 10 days after inoculation with SLS. (A) Laboratory rabbit infected with SLS at Day 10. The eyes are closed by a creamy mucopurulent discharge and there are secondary swellings around the nose (arrowhead) and at the base of the ears (arrow). In (B), a rabbit infected with Ur shows a similar range of signs with swollen inflamed eyelids and conjunctivae and swellings at the base of the ear and on the nose (arrow). However, there is much less discharge from the eyes and the eyes are open. (C) Wild rabbit infected with SLS at Day 10 with clinical signs similar to the laboratory rabbit infected with Ur. Wild rabbits infected with Ur have very few clinical signs at Day 10 (D). Wild rabbits infected with SLS became more severely affected until Day 12 and then began to recover. (E and F) The same rabbit as IC at Days 15 and 20, respectively.
and no mucopurulent discharge from nose or eyes (Figs. 1E and IF). Wild rabbits infected with Ur developed a very mild disease (Fig 1D) with the clinical signs mainly limited to the primary lesion which were 

$0.4 \times 2.5$ cm (length $\times$ breadth). Secondary lesions were not a prominent feature with only one rabbit having a discrete secondary lesion on an eyelid at 10 days after infection. Seven of nine rabbits developed mild anogenital swelling and scrotal edema by Day 10. At autopsy draining lymph nodes and spleens were enlarged two to three times normal size by Day 10, but contralateral nodes were not enlarged until Day 15.

Rectal temperatures were elevated from Day 5 after infection (Fig 2A). Temperatures decreased in the two laboratory rabbits infected with SLS between Days 8 and 10. Temperatures in wild rabbits infected with SLS remained $\geq 40^\circ$C until Day 13, rose again at Day 17, and then declined toward normal. The temperatures of laboratory rabbits infected with Ur followed a similar pattern but were not consistently $\geq 40^\circ$C until Day 10. Wild rab-

bits infected with Ur had a relatively mild temperature elevation, which had returned to normal about Day 17.

Peripheral blood leukocyte counts were increased in the SLS infected laboratory rabbits at 8 and 10 days after infection (Fig 2B). This was due to an increase in the numbers of circulating neutrophils with a reversal of the neutrophil:lymphocyte ratio from 30:60 to 70:20. For the other groups of animals there was little change in either the absolute or differential leukocyte counts over the course of the infection.

Myxoma virus titers in the skin

Virus was detected by plaque assay of the skin at the inoculation site from all groups at 2 days after infection (Figs. 3A and 3B). There was little difference in the titers between Ur and SLS in either wild or laboratory rabbits. The highest titers occurred by 6 days after infection in both wild and laboratory rabbits and reached $10^8$ pfu/g. In laboratory rabbits infected with Ur, titres decreased to $10^5–10^6$ pfu/g at Days 15 and 20. In wild rabbits, titers of both Ur and SLS remained high ($10^5–10^7$ pfu/g) at Days 15 and 20.

Replication of myxoma virus in the skin is critical for transmission, and secondary lesions are an important source of virus for dissemination. In these experiments, we measured virus titers in a skin site on the opposite hind foot to that inoculated. All of the inoculated laboratory rabbits and all the wild rabbits inoculated with SLS developed virus titers at this skin site (Figs. 3C and 3D). In all but one case, only laboratory rabbits inoculated with SLS had virus titers above the $10^7$ pfu/g transmission threshold in the distal skin. There was also a distinct difference between the virus types and the time at which virus was detected in the distal skin. SLS was present in distal skin of both laboratory and wild rabbits by 4 days after inoculation, but in Ur-inoculated laboratory rabbits, virus was not detected until Day 6. A small amount of virus was found in distal skin from wild rabbits inoculated with Ur at 15 and 20 days but only in one of three rabbits at each time point.

Replication of myxoma virus in the lymph nodes

In laboratory rabbits infected with SLS or Ur, virus was present in the draining popliteal lymph node by Day 2 with titres of $10^9$–$10^{10}$ pfu/g (Fig. 4A). For laboratory rabbits infected with SLS, virus titres were $>10^6$ pfu/g for five of the six rabbits harvested between Days 4 and 10 after infection. By comparison laboratory rabbits infected with Ur generally had lower virus titres in the draining lymph node with only one of six rabbits reaching titres of $>10^7$ pfu/g and only three of six having titres $>10^6$ pfu/g. Ur was cleared from the draining lymph node by 15 days after infection. One of two wild rabbits infected with SLS had detectable virus at Day 2 in the draining lymph node; however, at this time, virus was not detectable in the

**FIG. 2.** (A) Rectal temperatures of rabbits inoculated with myxoma virus. Temperatures were measured daily and are shown as the mean for all animals in each group that were alive at each time point (temperatures were always measured prior to killing). For clarity, error bars have been omitted. (B) Peripheral white cell counts for rabbits inoculated with myxoma virus. Total white cell counts per milliliter of blood are shown as the mean for each group of animals. For clarity, error bars have been omitted.
nodes from the Ur-infected wild rabbits (Fig. 4B). At 4 days after infection, all the wild rabbits infected with either SLS or Ur had virus present in the draining lymph node. In wild rabbits infected with SLS, virus titres in the draining node were \( \times 10^6 \) pfu/g. Although virus was detectable in the node until Day 20, titers were \( \times 10^5 \) pfu/g from Day 10 onwards. In general, wild rabbits had titres of SLS 10–100 times lower than laboratory rabbits. In wild rabbits, Ur reached titres of \( \times 10^4 \) pfu/g, but by Day 10 only one of three rabbits had detectable virus in the draining node and no rabbits had detectable virus after Day 10.

Virus titers were also measured in the popliteal lymph node from the uninoculated hind leg (contralateral lymph node) as a measure of dissemination to and replication in distal lymph nodes. In laboratory rabbits infected with SLS, virus was detected in the contralateral lymph node by Day 4 after inoculation. One of two laboratory rabbits infected with Ur had detectable virus in the draining node and no rabbits had detectable virus after Day 10.

Virus titers were generally 10–100 times lower than for laboratory rabbits (Fig. 4D).

**Myxoma virus in the spleen and peripheral blood mononuclear cells**

In the laboratory rabbits infected with SLS, virus was first detected in the spleen with titres \( \times 10^3 \) pfu/g at 4 days after inoculation. One of two laboratory rabbits infected with Ur had detectable virus in the spleen at this time. SLS reached a maximum titer of \( 5 \times 10^5 \) pfu/g at Day 10, whereas Ur was \( 8 \times 10^3 \) pfu/g at the same time point. Ur was not detected in the spleen after Day 10. In wild rabbits, SLS was also present in the spleen at Day 4 but only in one of three rabbits. At 6 and 10 days after infection, all three wild rabbits had SLS in the spleen with titers ranging from \( 10^3 \) to \( 5 \times 10^4 \) pfu/g. In one wild rabbit, SLS was still detectable in the spleen at Day 15, but it was not detected at Day 20. Wild rabbits inoculated with Ur had virus in the spleen at Days 6 and 10 only. Maximum titers were \( 3 \times 10^3 \) pfu/g (Fig. 5A).

Virus was detected in peripheral blood mononuclear cells of all SLS-infected laboratory rabbits from Day 4 to 10. Titers ranged from 5 to \( 10^5 \) pfu/10^9 cells (Fig. 5B). Virus was detected in the peripheral blood mononuclear cells of Ur-infected laboratory rabbits at Days 6 and 10 but only as an occasional plaque (detection level = 2 pfu/10^6
cells). Virus of either strain was only detected in blood mononuclear cells from one wild rabbit. This was at Day 6 from a rabbit infected with SLS and the titer was 22 pfu/10^6 cells.

Replication of myxoma virus in lung

Respiratory distress is a major clinical feature of myxomatosis. However, except in one case, the lungs appeared relatively normal at autopsy. Virus was not detected in lung tissue until Day 6, and the only infection to reach substantial titers in lung tissue was SLS in laboratory rabbits (Fig. 6). In all other groups, virus titers in the lungs were <10^3 pfu/g. Only two wild rabbits had detectable virus in the lungs, and this was at Days 6 and 10 after inoculation with SLS.

Serum antibody responses to myxoma virus

The serum IgM, IgG, and neutralizing antibodies were measured for each of the rabbits killed at each time point (Table 2). Antibody was detectable from Day 6 but SLS induced higher levels of neutralizing antibodies earlier than Ur in both wild and laboratory rabbits. Wild rabbits infected with SLS also produced much higher titers of IgG antibodies to myxoma virus than either laboratory or wild rabbits infected with Ur.

Cell permissivity to myxoma virus infection

To determine whether there was any difference in cell permissivity for myxoma virus replication in wild and laboratory rabbits, we prepared primary fibroblast cultures and primary lymphoid cell cultures from three wild and three laboratory rabbits and measured productive infection of these cultures by SLS in terms of the titers of virus produced following infection and the proportion of cells infected. In primary fibroblasts infected with SLS at an m.o.i. of 3, 95–100% of cells were infected as judged by immunofluorescence staining for virus in cells from both wild and laboratory rabbits. The virus titers (log_{10} mean ± SE pfu/10^6 cells) in fibroblasts at 24 h were 6.2 ± 0.3 for wild rabbits and 5.9 ± 0.4 pfu for laboratory rabbits; there was little difference in these titers at 48 and 72 h (data not shown). In primary lymphoid cells from either the lymph nodes or spleens, ~50% of the cells were infected from either rabbit type at 24, 48, and 72 h.

FIG. 4. (A) Virus titers in the draining lymph node of laboratory rabbits infected with SLS (●) or Ur (○). The titers for each rabbit at each time point are shown as a scatterplot. In addition, the geometric mean titers have been plotted separately for SLS (solid line) and Ur (dashed line). (B) Virus titers in the draining lymph node of wild rabbits infected with SLS (●) or Ur (○). (C) Virus titers in the contralateral node of laboratory rabbits infected with SLS (●) or Ur (○). (D) Virus titers in the contralateral lymph node of wild rabbits infected with SLS (●) or Ur (○).
after infection, suggesting that there was a subset of cells insusceptible to infection. Virus titers in cells from lymph nodes were $5.5 \pm 0.4$ in wild rabbits and $5.6 \pm 0.4$ in laboratory rabbits at 24 h and similar titers occurred at 48 and 72 h. The titers in lymphoid cells prepared from the spleens were similar to those in cells prepared from the lymph nodes.

**DISCUSSION**

The pathogenesis of myxomatosis was examined at two levels. Firstly, the differences in virus replication between the attenuated Ur strain of myxoma virus and the virulent SLS were examined in laboratory rabbits. These rabbits have never been selected for resistance to myxomatosis and serve as a model for the Australian wild rabbit population in 1950 when SLS was released. We then compared virus replication in these laboratory rabbits with the same virus strains in rabbits from the wild population, which have undergone selection for resistance to myxomatosis for nearly 50 years. Because the genetics of the viruses were held constant, this allowed direct comparison of the two rabbit populations.

The use of two virus strains of markedly different virulences provided a demonstration of the degree of genetic resistance that has developed in the wild rabbit population.

The low doses of virus (100 pfu), comparable to what a mosquito might deliver (Day et al., 1956), and the selection of an inoculation site that was largely drained by a single lymph node allowed a detailed examination of the steps involved in viral dissemination, control, and clearance. Some care needs to be taken in interpreting these data. Firstly, outbred animals were used, and these would not all have the same level of resistance to myxoma virus. Secondly, the scale of the experiments meant that limited numbers of animals were used at each time point, thus single time points may have a large variance. However, the use of laboratory and wild rabbits and virulent and attenuated virus strains at each time point provided a very clear picture overall of where virus replication was controlled.

Resistance to myxomatosis was not an absolute event but reflected a dialog between the host and virus. In laboratory rabbits infected with an attenuated virus or in wild rabbits, the advantage was shifted toward the host. Thus in laboratory rabbits infected with Ur, the severity of the clinical signs was reduced and their onset was slower although the actual clinical signs were similar to SLS infected rabbits. Similarly, wild rabbits infected with SLS became quite ill, particularly between 10 and 15 days after infection, but then recovered quickly from Day 15. Wild rabbits infected with Ur did not become ill and clearly were able to control the infection.

The rectal temperatures of laboratory rabbits infected with SLS fell in the final 48 h of the experiment. This was accompanied by an increased white blood cell count predominantly due to a neutrophilia. These signs were not seen in the otherwise quite ill laboratory rabbits infected with Ur or in the wild rabbits infected with SLS.
Both of these groups maintained elevated rectal temperatures, and recovery from myxomatosis was accompanied by a gradual return to normal rectal temperatures. During the initial stages of SLS replication, there was no difference in rectal temperatures in wild or laboratory rabbits. This demonstrated that an elevated body temperature was not sufficient to control myxoma virus replication in tissues distal to the skin. In contrast, replication of myxoma virus on chick chorioallantoic membranes was inhibited at temperatures over 39°C (Marshall, 1959), and there is some evidence that attenuated strains of myxoma virus replicated less efficiently than virulent strains at high temperatures in tissue culture (Ross and Sanders, 1979).

The replication and dissemination of SLS in laboratory rabbits in these trials was similar to that described by Fenner and Woodroofe (1953). Thus in our trials, virus was present in the skin at the inoculation site and in the lymph node draining this site by 48 h after inoculation. At 4 days, it was present in the contralateral lymph node, spleen, peripheral blood mononuclear cells, and distal skin. By Day 6, it was detectable in the lung. As previously suggested (Fenner and Woodroofe, 1953), this is compatible with a spread in infected lymphocytes in the lymph to the blood and hence to distal lymphoid tissues as lymphocytes traffic through these and also to skin and other tissues.

All groups of rabbits developed similar titers of virus in the skin at the inoculation site. These titers were sufficient for both SLS and Ur to have been transmitted by mosquitoes from the primary lesions in wild and laboratory rabbits (Fenner et al., 1956). The similarity in titers indicated that neither attenuation nor resistance were initially strongly manifested within the skin. However, histological studies showed that wild rabbits had a much more pronounced inflammatory response than laboratory rabbits by Day 10 even though virus titers were similar (S. M. Best and P. J. Kerr, in preparation) Resistance was very evident at the distal skin site. In laboratory rabbits, SLS replicated to high titers in the distal skin, whereas in wild rabbits titers of SLS were 100-fold lower. The attenuated Ur reached the distal skin later than SLS and replicated to lower titers in both wild and laboratory rabbits. The fact that the virus reached the distal skin site but then was restricted in its amplification suggests that there was a functional immune response operating at this site between Days 4 and 6. Interestingly, apart from one wild rabbit at one time point, titers in the distal skin were too low for virus transmission to have occurred except from SLS-infected laboratory rabbits.

The earliest evidence of control of virus replication in wild rabbits compared to laboratory rabbits was in the lymph node draining the inoculation site. In wild rabbits, both SLS and Ur had 10- to 100-fold lower titers in this node, as early as Day 4, compared to the same viruses in laboratory rabbits. Although there was a suggestion that Ur replicated less efficiently than SLS in the draining node of laboratory rabbits, the difference between SLS and Ur was quite clear in wild rabbits. The differences in virus titers and time of detection in distal skin, contralateral lymph node, and lung between wild and laboratory rabbits and virulent and attenuated viruses indicated that the draining lymph node was a critical organ for amplification and dissemination of myxoma virus. It appeared that early control of myxoma virus replication in this tissue rather than the skin was critical for the disease outcome.

Despite differences in titers in tissues of wild and laboratory rabbits, SLS replicated equally efficiently in both lymphoid cells and fibroblasts prepared from laboratory and wild rabbits. Thus the reduced titers of virus in the lymph nodes and other tissues of wild rabbits were not due to resistance of the wild rabbit cells to virus replication. This implies that control of infection was due to a more effective immune response in wild rabbit lymph nodes and subsequent tissues compared to laboratory rabbits. This must become effective between 2 and 4 days after infection when the lower titers of virus were first observed. The virulent SLS was still able to persist in the lymph nodes of some wild rabbits for the duration of

### Table 2

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<th>Day</th>
<th>Laboratory rabbits</th>
<th>Wild rabbits</th>
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<tr>
<td></td>
<td>SLS IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>—</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>10</td>
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<td>2.4</td>
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<td>15</td>
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<td>20</td>
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<sup>a</sup> Antibody was not detectable before Day 6.<br>
<sup>b</sup> Geometric mean log 10 titers for the two laboratory or three wild rabbits killed at each time point.

RESISTANCE TO MYXOMA VIRUS IN EUROPEAN RABBITS

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**TABLE 2**

Serum Antibody IgM and IgG Antimyxoma Virus Titers Measured by ELISA and by Neutralization Assay (N) in Laboratory and Wild Rabbits Infected with either SLS or Ur
the experiment, suggesting that it was able to suppress or avoid this response to some extent. However, the attenuated Ur virus was quickly cleared from the wild rabbit lymph nodes with only one of three rabbits having detectable virus at Day 10 after infection.

Conceptually myxomatosis has been compared with mousepox (Fenner and Woodrofe, 1963). Recovery from mousepox virus infection in inbred strains of mice depends on effective innate effectors such as NK cells, macrophages, nitric oxide, and type I and type II interferons, as well as adaptive CTL responses (which require antigen specific T helper cells producing type-1 cytokines such as interferon-γ) and antibody (O'Neill and Brenan, 1987; Jacoby et al., 1989; Karupiah, 1998; Karupiah et al., 1993a,b, 1996). In particular, a Th1 cytokine profile in lymph nodes (interferon-γ, IL-2, IL-12, and IL-4) was correlated with a rapid and effective antiviral response whereas a Th 2 profile (IL-4) led to failure to control the infection and death (Karupiah, 1998). In addition, clearance of virus from the skin requires a different subset of effector cells to that required for clearance from other tissues (Karupiah et al., 1996). Similar results have been obtained for other viruses in outbred animals; for example, macaques infected with an attenuated simian immunodeficiency virus had a Th1 cytokine profile in the lymph node, whereas those infected with a virulent SIV had a Th2 profile (Zou et al., 1997).

In a simple conceptual model based around mousepox, resistance to myxomatosis in wild rabbits could be explained by selection for an enhanced innate immune response to myxoma virus. Thus in resistant rabbits, the virulent virus would not effectively suppress the initial inflammatory response to infection even though the virus produces the same modulators of the immune response that induce a lethal infection in laboratory rabbits. This would allow antiviral mediators such as interferons and TNF-α to induce an antiviral immune response, which, between 2 and 4 days after infection, limited the amplification of the virus at the draining lymph node. Development of an NK cell response at this site and in other tissues would further limit viral replication and enhance antiviral cytokine production. Although some researchers have suggested that laboratory rabbits do not have detectable NK cell activity (Laybourn et al., 1990), others have shown in vitro killing of vaccinia virus infected xenogeneic targets (Woan et al., 1978), suggesting that NK cell activity does occur in rabbits and therefore that these cells are likely to be important in control of virus infections.

Limiting the amplification of SLS and the associated immunosuppressive activities of the virus would allow a cell mediated response specific for myxoma virus to develop, and this, as well as the innate, nonspecific antiviral response, could cause the effective control of virus seen in distal lymphoid tissues, skin and lung of wild rabbits. Because virus antigens will be presented to effector T cells earliest in the draining lymph node, we suggest that events in this node are critical for development of a T cell response for controlling virus replication in other tissues.

Infection of laboratory rabbits with Ur provides a useful contrast to SLS. In our model, these laboratory rabbits have not been selected for the development of a strong innate response, but the lower virulence of the virus may enable the animal to survive long enough to mount an effective immune response. The fact that lower virus titers were not seen at the primary inoculation site in the skin would fit with the evolution of myxoma virus to replicate predominantly within the skin of its native rabbit hosts rather than inducing a lethal disseminated infection (Fenner and Ratcliffe, 1965). Thus the virus is more effective at evading the host immune response within the skin at the inoculation site rather than distally in other tissues. An interesting point is that Ur was slower to reach the draining lymph node in wild rabbits, suggesting that it may also disseminate less efficiently from the inoculation site in these rabbits. This raises the questions of whether Ur then disseminates less efficiently to other tissues, whether the control of replication within the draining lymph node prevents this dissemination, or whether the development of an immune response simply controls replication in tissues distal to the node. Further studies are needed to differentiate between these possibilities.

This highly simplified model makes specific, testable predictions that will require the development of assays for rabbit cytokines and cellular effectors. At this stage, we have only examined antibody production in the immune response to myxoma virus infection. Antibody production was not a good prognostic indicator as SLS-infected laboratory rabbits produced IgM and IgG and had neutralizing activity by 10 days after infection but would all have died. However, this showed that isotype switching was occurring and thus that there was presumably some T helper cell activity. The production of high levels of antibodies by laboratory rabbits infected with SLS could imply that the CD4 T cell response has been predominantly Th2, dominated by IL-4, rather than an antiviral Th1, dominated by interferon-γ. However, wild rabbits infected with SLS also produced high titers of antibodies. In our proposed model, this could be explained by production of interferon-γ and a Th1 response in wild rabbits. In a murine model, this would lead to production of IgG2a and IgG3 rather than the Th2 induced IgG1 (Abbas et al., 1996). However, unlike species such as rodents and humans, rabbits do not produce different IgG subclasses, in response to different Th cytokine stimuli, as only one C-γ heavy chain gene is present (Knight and Crane, 1994), thus it was not possible to test whether a predominantly Th1 or Th2 response was occurring based on the IgG subclasses.

The genetics of resistance to myxomatosis in rabbits...
have not been investigated, but more than one locus is likely to be involved (Kerr and Best, 1998). In mice, strain-dependent resistance to mousepox is multigenic. The main resistance locus Rmp-1 maps to the NK cell complex on mouse chromosome 6 and may be identical to Cmv-1, which encodes resistance to murine cytomegalovirus (Brown et al., 1997). Whether Australian wild rabbits have enhanced resistance to other virus infections or an enhanced propensity to develop such resistance and whether this resistance to myxomatosis leaves the rabbit more vulnerable to other parasites such as protozoa or helminths are unresolved questions. The lethality of the calicivirus causing rabbit hemorrhagic disease for both laboratory and wild rabbits in Europe and Australia suggests that resistance is likely to be myxoma virus specific. The specificity of resistance could be examined by testing the resistance of Australian wild rabbits to other poxviruses such as the vaccinia virus-derived rabbitpox virus.

At this stage, the genetic differences between Ur and SLS are not known. Restriction mapping of Ur and the SLS derived strain of myxoma virus “Glenfield” did not reveal any restriction fragment length polymorphisms (Russell and Robbins, 1999). Subsequent studies have demonstrated that restriction maps of Glenfield and SLS are identical but SLS could be distinguished from Ur by a HaeIII polymorphism (P. J. Kerr and K. M. Saint, in preparation). The conservation of the restriction maps indicates that large scale genetic rearrangements have not occurred in Ur but the genetic basis of attenuation remains to be determined.

MATERIALS AND METHODS

Viruses

The Standard Laboratory Strain of myxoma virus (SLS) used in this study was derived from a freeze-dried rabbit tissue stock prepared by Professor Frank Fenner (John Curtin School of Medical Research, Canberra, Australia) in 1953. This was subsequently passaged twice in RK13 cells and twice in rabbits. The virus is of grade 1 virulence (Fenner and Marshall, 1957), killing 100% of infected rabbits with an average survival time of <13 days, and this was confirmed prior to this study (Robinson et al., 1999). The Uriarra (Ur) strain of myxoma virus was derived from the Uriarra/2/53–1 isolate (Mykytowycz, 1953) by Russell and Robbins (1989). It was passaged an unknown number of times in CV-1 cells before we obtained it and subsequently was passaged twice in Sirc cells and twice in rabbits. This virus is of grade 5 virulence, and >95% of laboratory rabbits recover from infection but all develop clinically severe myxomatosis (Fountain et al., 1997; Kerr unpublished data).

Virus stocks were prepared as homogenates from testes harvested from laboratory rabbits 8–9 days after intradermal inoculation with either SLS or Ur. Titers were determined by plaque assay on Vero cell monolayers.

Tissue culture

Vero cells were cultured in minimal essential medium (MEM, Gibco BRL) supplemented with 10% newborn bovine serum, 2 mM L-glutamine, 200 units/ml penicillin, and 100 μg/ml streptomycin. Rabbit fibroblasts were produced by explant outgrowth from pieces of tunica vaginalis. Fibroblasts were cultured in MEM supplemented as above except that initially 25% fetal bovine serum was used, and this was reduced to 10% after 5 days. Primary lymphoid cells were prepared from lymph nodes and spleens by passing the minced tissues through mesh screens (Kruisbeck, 1993). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 200 units/ml penicillin, and 100 μg/ml streptomycin. Cell viability was assessed by trypan blue exclusion and was usually >95%.

Infection of primary fibroblasts and lymphoid cells from wild and laboratory rabbits

Primary fibroblasts were prepared from three wild and three laboratory rabbits. SLS was inoculated at a m.o.i. of 3. There were three replicates for each rabbit at each time point. The cells were harvested at 24, 48, and 72 h and plaque assayed as for other tissues. Primary lymphoid cells, prepared separately from the popliteal lymph nodes, and the spleens of the same rabbits used for fibroblast cultures were infected with SLS at a m.o.i. of 3, three replicates from each rabbit at each time point. At 24, 48, and 72 h, wells were harvested and virus titers were determined by plaque assay.

To determine the proportion of cells infected, fibroblasts from each rabbit were seeded onto sterile glass coverslips in 24-well plates and infected as before. At 24, 48, and 72 h, the cells were fixed for 5 min in ice-cold acetone/methanol (1:1) at 4°C, washed twice in PBS, blocked with 3% BSA/PBS at room temp for 60 min, and incubated for 2 h at 37°C with 100 μl of a 1:200 dilution, in 1% BSA/PBS, of monoclonal antibody 3B6E4, which stains myxoma virus antigen in infected cells (Fountain et al., 1997). Antibody binding was visualized by the addition of goat anti-mouse IgG conjugated to FITC (Boehringer Mannheim) and viewed with confocal microscopy. For lymphoid cells, the same process was followed except the cells were harvested, fixed, and then transferred onto poly-L-lysine coated slides using a cytopsin.

Rabbit breeding and housing

All animal experiments followed CSIRO/NHMRC guidelines for animal usage and were approved by the Gunaghlin animal experimentation ethics committee.
Laboratory rabbits were bred at the Gungahlin animal facility and were an outbred line of predominantly New Zealand White stock. Wild rabbits were bred in the Gungahlin animal facility, and the breeding stock was derived from rabbits captured as kittens in the Canberra district. The animals used for experiments were one to two generations removed from the wild. There was no selection for resistance to myxomatosis in the breeding facility.

For experimental use all rabbits were housed in individual cages in a temperature and light controlled animal room. Standard rabbit pellets and water were provided ad libitum and green vegetables were supplied weekly. Shelters were provided in each cage as a refuge for the wild rabbits.

Only male rabbits were used in the experiments, and all rabbits were >4 months old.

Indian ink studies to establish draining lymph node

The popliteal lymph node was confirmed as the predominant node draining the inoculation site by injection of 500 μl of Indian ink intradermally into the skin over the metatarsal bones of both hind feet of three rabbits. The rabbits were killed 24 h later, and the popliteal, inguinal and prefemoral lymph nodes were dissected from each leg and examined for ink staining. In five of six inoculations, the popliteal node was the only node stained, in one case the popliteal node was heavily stained and there was light staining on the cut surface of the inguinal node. A similar experiment was performed for intradermal inoculation of the skin over the midpoint of the biceps femoris muscles of the thigh. In this experiment, the major drainage was also to the popliteal nodes, but there was staining of the inguinal node in two of the six inoculations and the prefemoral node in a separate two of the six inoculations. The lymph node drainage was further examined by inoculation of SLS and Ur strains of myxoma virus at each site and the virus titers measured in the left and right popliteal lymph nodes and spleens. This confirmed that the foot inoculation provided good stepwise drainage of virus to the popliteal node, but that virus inoculated into the thigh did not reach this node directly from the skin site but was found in equal titers in left and right popliteal nodes and spleen at 4–6 days after infection.

Infections and monitoring of rabbits

Laboratory rabbits were inoculated with 100 pfu of either SLS (8 rabbits) or Ur (12 rabbits) intradermally into the skin over the metatarsal bones of the left hind foot. Rabbits were examined daily and clinical signs and rectal temperatures recorded. Wild rabbits were inoculated in the same way, but 16 rabbits were inoculated with each virus. Two laboratory rabbits infected with SLS were killed at each of 2, 4, 6, and 10 days after inoculation. For wild rabbit infections, two rabbits were killed at 2 and 4 days after inoculation and three at 6, 10, 15, and 20 days after inoculation. Rabbits were randomly allocated to killing times prior to inoculation and killed by an intravenous overdose of barbiturate.

Tissue collection and processing

Approximately 25 ml of blood was collected by cardiac puncture into a heparinized syringe immediately following death. At autopsy the skin inoculation site, the left popliteal lymph node (draining lymph node), right popliteal lymph node (contralateral node), skin from the equivalent site on the right hind foot to the inoculation site (distal skin), spleen, and anterior lobes of the lungs were collected. The tissues were trimmed to remove fat, divided as necessary for further analysis, and weighed. Lymph nodes, spleens, and lung samples were then chopped finely with scissors or scalpels and homogenized using a small pestle in a 1.5-ml plastic tube. Tissue homogenates were suspended in MEM as a 10 or 25% w/v suspension, sonicated (3 × 5 s cycles on ice) using a probe sonicator and freeze thawed twice. Skin samples were shaved of hair, washed in PBS, minced with a scalpels blade and homogenized using a small pestle and a 1.5-ml plastic tube. Tissue homogenates were suspended in 0.1 mg/ml collagenase D (Boehringer Mannheim) and incubated at 37°C for 90 min with frequent agitation. This was followed by sonication and freeze thawing as for other tissues. The collagenase digestion was optimized using different concentrations of collagenase and incubation times and did not reduce titers of virus when uninfected skin samples were incubated with either 10³ or 10⁴ pfu of added virus.

Peripheral blood mononuclear cells were prepared from blood collected when the rabbit was killed. The whole blood was centrifuged (500 g, 20 min), serum was removed and stored at −20°C for subsequent assay, and the buffy coat was collected and resuspended in 4 ml of PBS. This was layered over 3 ml of Ficoll–Paque (Pharmacia) and centrifuged (400 g, 20 min). The mononuclear cell layer at the Ficoll/PBS interface was collected and washed twice in MEM before being counted and stored at −70°C for subsequent plaque assay.

Virus titrations

Virus titers in tissue suspensions were measured by plaque assay in duplicate on Vero cell monolayers. Titers were expressed as the mean of the duplicate determinations in pfu/g of tissue or for peripheral mononuclear cells as pfu/10⁶ cells. Limits of detection were 100 pfu/g or 1–2 pfu/10⁶ cells.
ELISA

ELISA for IgM and IgG antibodies to myxoma virus were performed using twofold serum dilutions from 1:50 (Kerr, 1997). Endpoint titers were defined as the reciprocal of the dilution giving an OD (405 nm) 0.1 units above the 1/100 dilution of the negative serum.

Plaque reduction neutralization assays

Plaque reduction neutralization assays were performed on Vero cell monolayers as previously described (Kerr, 1997) using constant virus concentration (≈100 pfu) and twofold dilutions of heat inactivated serum (30 min, 56°C). Titers were the reciprocal of the lowest dilution giving a ≥50% reduction of the number of plaques obtained with normal serum.

Leukocyte counts and differential counts

Peripheral blood samples (0.5 ml) were collected from the marginal ear vein of all rabbits every 48 h, diluted 1:10 in an aqueous solution of 2% (v/v) glacial acetic acid and 0.01% (w/v) gentian violet, and leukocytes counted using a hemocytometer. Differential leukocyte counts were made on air dried films stained with Wright's stain.

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